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First Record of the Tree-frog Genus *Liuixalus* from Vietnam with the Description of a New Species (Amphibia: Rhacophoridae)

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Abstract: We record a tree frog of the genus *Liuixalus* for the first time from outside of China and describe it as a new species, *Liuixalus catbaensis*, on the basis of a single juvenile specimen collected from Cat Ba Island, northern Vietnam. The new species is easily distinguished from all other members of the genus *Liuixalus* by its uniformly brick-red dorsum lacking dark markings. The biogeographical significance of finding of this species in Vietnam is briefly discussed.

Key words: *Liuixalus catbaensis*; Mitochondrial phylogeny; Southeast Asia; Taxonomy; Zoogeography

Introduction

The Old-World tree-frog genus *Liuixalus* Li, Che, Bain, Zhao, and Zhang, 2008 currently contains three species *L. romeri* (Smith, 1953), *L. ocellatus* (Liu and Hu in Liu, Hu, Fei, and Huang, 1973), and *L. hainanus* (Liu and Wu, 2004) occurring in southeastern China (e.g., Fei et al., 2009). These species were formerly placed in various genera, *Philautus* Gistel, 1848, *Chirixalus* Boulenger, 1893, *Chiromantis* Peters, 1854, and *Aquixalus* Delorme, Dubois, Grosjean, and Ohler, 2005 (Smith, 1953; Liu et al., 1973; Bossuyt and Dubois, 2001; Frost et al., 2006; Fei et al., 2010). However, based on results of recent molecular studies on rhacophorids (Li et al., 2008), they were moved to a distinct genus, *Liuixalus*, with *L. romeri* as the type species.

Although Frost (2013) listed Vietnam in the distribution range of the genus *Liuixalus*, no formal report of occurrence of the genus has been given outside Southeast China. However, a juvenile specimen of a small rhacophorid species collected in our recent field survey in northern Vietnam (Fig. 1) proved to be a member of the genus *Liuixalus* through subsequent molecular analysis. The specimen formed a clade on mtDNA trees with members of the genus, but was placed in a unique position different from available sequences of its congeners. Because the specimen also differed morphologically from all the known members of *Liuixalus*, we describe it as a new species of the genus. This is also the first record of the genus outside China.

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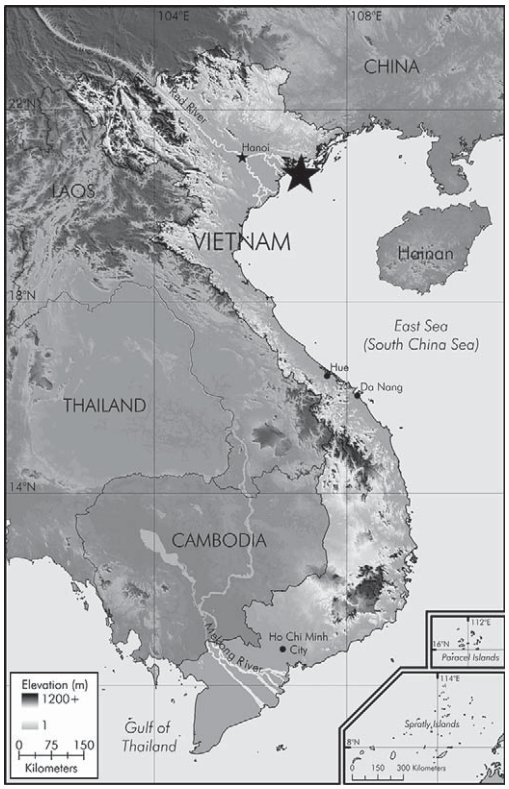

FIG. 1. Map of Vietnam showing Cat Ba Island (filled star), where the rhacophorid sp. was found.

MATERIALS AND METHODS

Specimens examined are stored in the collection of the Vietnam National Museum of Natural History (VNMN) and Chengdu Institute of Biology (CIB). We obtained DNA sequence data from tissues preserved in 99% ethanol for a specimen of rhacophorid species from Vietnam (VNMN 3684=TH [Tsutomu Hikida's field number] 2155; GenBank accession number AB871420). Methods for DNA extraction, and amplification and sequencing of the mtDNA fragments are the same as those reported by Kuraishi et al. (2013). We deposited the resultant sequence (858 base pairs [bp] of partial sequences of mitochondrial 16S rRNA gene) in GenBank (Accession numbers as shown above).

In order to generically assign the rhacophorid specimen from Vietnam, we first compared a partial sequence of 16S rRNA (ca. 800 bp) with GenBank data of rhacophorid species whose congeners are recorded from Vietnam (Orlov 2012), i.e., *Chiromantis hansenae* (Cochran, 1927) (AB813161), *Feihyla kajau* (Dring, 1984) (AB847122), *Gracixalus gracilipes* (Bourret, 1937) (DQ283051), *Kurixalus appendiculatus* (Günther, 1859) (AB847125), *Liuixalus romeri* (EU215528), *Nyctixalus pictus* (Peters, 1871) (DQ283133), *Philautus abditus* Inger, Orlov, and Darevsky, 1999 (GQ285673), *Polypedates leucomystax* (Gravenhorst, 1829) (AB728138), *Rhacophorus kio* Ohler and Delorme, 2006 (AB781695), *Rhaorchestes parvulus* (Boulenger, 1893) (AB871421), *Theloderma leprosum* Tschudi, 1838 (AB847128), and *Buergeria buergeri* (Temminck and Schlegel, 1838) (AB127977). Following the result obtained (see results), we then determined the relationships of the specimen from Cat Ba Island, Vietnam, by comparing the partial sequence of 16S rRNA (ca. 500 bp) with those of *Liuixalus romeri* from Hong Kong (CIB [Chengdu Institute of Biology] 20080048; AB871412) and *L. ocellatus* from Wuzhishan, Shuiman, and Diaoluoshan, Hainan Island, China (CIB 20081062, 63, 65–69; AB871413–871419), and additional GenBank data (*L. hainanus* [GQ285671, KC465826], *L. ocellatus* [GQ285672, GU120328, KC465829] and *L. romeri* [EF564535, EF564536]), as well as *Chiromantis hansenae* (AB813161) and *Buergeria buergeri* (AB127977) as out group. For tree construction and calculation of genetic distances (uncorrected p-distance), we followed Kuraishi et al. (2013).

We took the following 32 body measurements to the nearest 0.1 mm with a dial caliper under a binocular microscope, and to the nearest 0.01 mm using a micrometer, mainly following Matsui (1984, 1994): (1) snout-vent length (SVL); (2) head length (HL) from tip of snout to hind border of angle of jaw, not measured parallel with the median line; (3) snout-nostril length (S-NL); (4) nostril-eyelid length (N-EL); (5) snout length (SL); (6) eye length (EL); (7) eye diameter (ED), diameter of the exposed portion of the eyeball; (8)

tympanum-eye length (T-EL); (9) tympanum diameter (TD); (10) head width (HW); (11) internarial distance (IND); (12) interorbital distance (IOD); (13) upper eyelid width (UEW); (14) forelimb length (FLL); (15) lower arm and hand length (LAL); (16–19) first to fourth finger disk diameters (1–5FDW); (20) inner palmar tubercle length (IPTL); (21) outer palmar tubercle length (OPTL); (22) hindlimb length (HLL); (23) thigh length (THIGH); (24) tibia length (TL); (25) foot length (FL); (26) first toe length (1TOEL); (27) inner metatarsal tubercle length (IMTL); (28–32) first to fifth toe disk diameters (1–5TDW). We followed the system of description of toe-webbing states used by Savage (1975).

Systematics

In the preliminary analyses using all known 11 genera from Vietnam and an outgroup *Buergeria*, the rhacophorid sp. from northern Vietnam formed a clade with *Liuixalus romeri* with full support (Bayesian posterior probability [BPP] = 1.00, bootstrap support [BS] = 100%). Rhacophorid sp. from Vietnam substantially differed from species of the ingroup genera (16.3% from *Gracixalus* to 22.6% from *Kurixalus*) other than *Liuixalus* (2.4%). These results clearly suggest placement of the rhacophorid sp. from Vietnam in the genus *Liuixalus*.

After adding sequences both from our own data and from GenBank, support for the monophyly of *Liuixalus* including the rhacophorid sp. from Vietnam was sufficient (BPP = 1.00, BS = 99%: Fig. 2), but the relationships within the clade were not resolved and five groups (*L. hainanus*, *L. romeri* from Jinxiu, Gungxi, the rhacophorid sp. from Vietnam, *L. romeri* from Hong Kong and Shiwanshan, Guangxi, and *L. ocellatus*) were recognized only in a maximum likelihood (ML) tree. The rhacophorid sp. from Vietnam was close to *L. romeri* from Jinxiu, Guangxi (EF564535, EF564536), but their monophyly was not supported (BPP = 0.53, BS = 35%).

The rhacophorid sp. from Vietnam differed from the species of *Liuixalus* by small genetic distances (2.2–3.0%). Because the distances

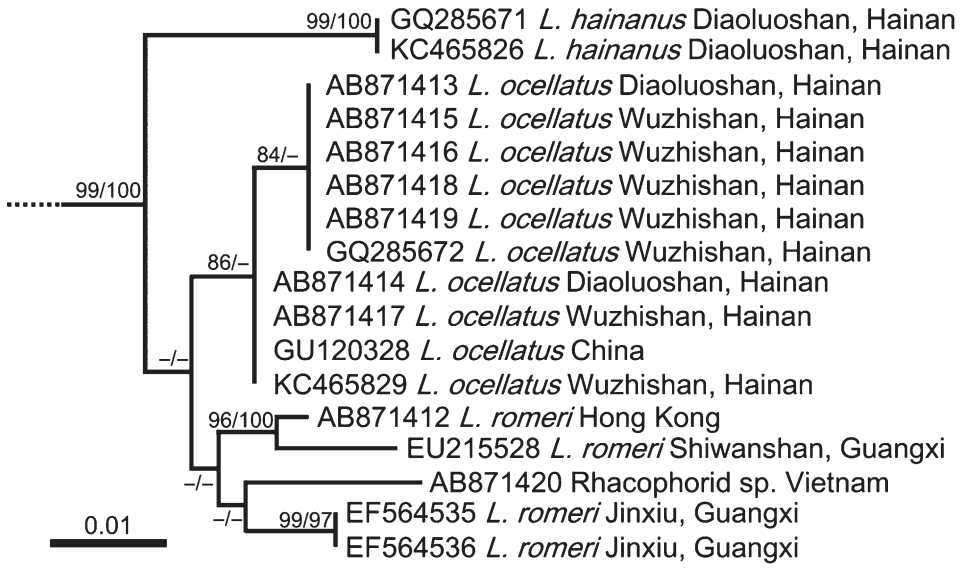

Fig. 2. Maximum-likelihood (ML) tree from ca. 500 bp partial sequence of mitochondrial 16S rRNA gene for the rhacophorid sp. from Vietnam and *Liuixalus*. A rhacophorine *Chiromantis hansenae* and a buergeriine *Buergeria buergeri* were used as outgroup. Numbers above or below branches represent bootstrap supports for ML inferences and Bayesian posterior probabilities (ML-BS/BPP).

between the three species of *Liuixalus* were also small, ranging from 1.8 to 3.0%, the rhacophorid sp. from Vietnam can be genetically regarded as a good species. Furthermore, it is also clearly separated morphologically from all nominal species of *Liuixalus*. Thus, we conclude that the rhacophorid sp. from Vietnam is a distinct species in the genus *Liuixalus* and describe it as follows.

Liuixalus catbaensis sp. nov.
(Figs. 3–5)

Diagnosis

The new species is assigned to the genus *Liuixalus* based on molecular evidence, and has morphological characteristics common to its congeners: intercalary cartilage present between terminal and penultimate phalanges of digits; tips of digits expanded into large discs bearing circum-marginal grooves; fingers free of webbing; toes very poorly webbed; no web between outer metatarsals; vomerine teeth absent. *Liuixalus catbaensis* is distinguishable from all its congeners by the brick-red dorsum lacking dark markings.

Etymology

The specific name refers to the Cat Ba Island of northern Vietnam, where the new species was found.

Holotype

VNMN 3684 (formerly TH 2155), unsexed juvenile, collected by An Thi Hang, between 9:00–13:00 h on 18 September 2013 from Ao Ech (20°47'56" N, 106°59'52" E; ca. 45 m a.s.l.) of the Cat Ba National Park, Cat Ba Island, Haiphong City, northern Vietnam.

Fig. 3. Juvenile holotype (VNMN 3684) of *Liuixalus catbaensis* sp. nov. in life.

Description of holotype

Unsexed juvenile; SVL 12.8 mm; head longer (HL 5.3 mm, 41.5%SVL) than wide (HW 5.1 mm, 39.8%SVL), wider than body; snout rounded in dorsal and lateral view, length (SL 2.3 mm, 18.1%SVL) longer than eye length (EL 2.2 mm, 17.2%SVL, ED 1.9 mm, 14.6%SVL), projecting beyond mouth; canthus rostralis clear; loreal region vertical and concave; nostrils slightly nearer to eye (N-EL 1.0 mm, 7.8%SVL) than to tip of snout (S-NL 1.1 mm; 8.4%SVL); internarial distance (IND 1.8 mm, 13.9%SVL) greater than interorbital distance (IOD 1.6 mm, 12.7%SVL) and upper eyelid (UEW 1.2 mm, 9.2%SVL); eye large, protuberant; pupil horizontal; tympanum distinct, circular, diameter (TD 0.8 mm, 6.4%SVL) about one-third of eye length and separated from eye by one-third of tympanum diameter (T-EL 0.2 mm, 1.8%SVL); vomerine teeth absent; choana oval; tongue notched posteriorly; vocal sac absent.

Forelimb slender (FLL 7.8 mm, 61.2%SVL); hand and forearm long (LAL 6.2 mm, 48.6%SVL); finger length formula: I<II<IV<III (Fig. 5A); expanded disks each with a circum-marginal groove and a transverse ventral groove; disks on third finger (3FDW 0.38 mm, 3.0%SVL,) slightly wider than those on second (2FDW 0.35 mm, 2.7%SVL) and fourth (4FDW 0.33 mm, 2.6%SVL), and much wider than first (1FDW 0.24 mm, 1.9%SVL), all much narrower than tympanum; webbing between fingers and lateral fringes on fingers absent; subarticular tubercles moderately developed; inner palmar tubercle flat (IPTL 0.39 mm, 3.0%SVL), outer one much smaller (OPTL 0.23 mm, 1.8%SVL); nuptial pad absent.

Hindlimb moderately long (HLL 19.5 mm, 152.3%SVL); tibiotarsal articulation reaching to point between eye and nostril when fully stretched leg adpressed to body; heels touching

each other when thigh (THIGH 6.8 mm, 53.2%SVL) and tibia (TL 6.9 mm, 53.6%SVL) placed at right angle to body; foot (FL 5.3 mm, 41.7%SVL) much shorter than tibia; toe length formula I<II=V<III<IV; toe tips expanded into enlarged disks each with a circummarginal groove and a transverse ventral groove; width of fourth and third toe disks (4TDW 0.41 mm, 3.2%SVL; 3TDW 0.39 mm, 3.1%SVL) greater than that of finger disks and remaining toe disks (1TDW 0.25 mm, 2.0%SVL; 2TDW 0.30 mm, 2.4%SVL; 5TDW 0.32 mm, 2.5%SVL); webbing formula I 2+–2++ II 2+–3++ III 2½–4 IV 3⅓–1¾ V (Fig. 5B); subarticular tubercles rounded; inner metatarsal tubercle small and oval, (IMTL 0.39 mm, 3.0%SVL), less than half the length of first toe (1TOEL 0.99 mm, 7.7%SVL); no outer metatarsal tubercle.

Dorsal surface nearly smooth, sparsely scattered with minute, blunt tubercles; strongly developed supratympanic fold between eye and arm insertion; flank scattered with flat tubercles; ventral surface smooth on lower jaw and breast, composed of flat granules on abdomen; no protruding tubercle at heel.

Color

In life, dorsal ground color on body brick-red with a few faint brown spots (Figs. 3, 4A); narrow dark stripe between eye and nostril below canthus; lips barred with white and black; side of head from snout and below eye to temporal region dusted with brown; darker

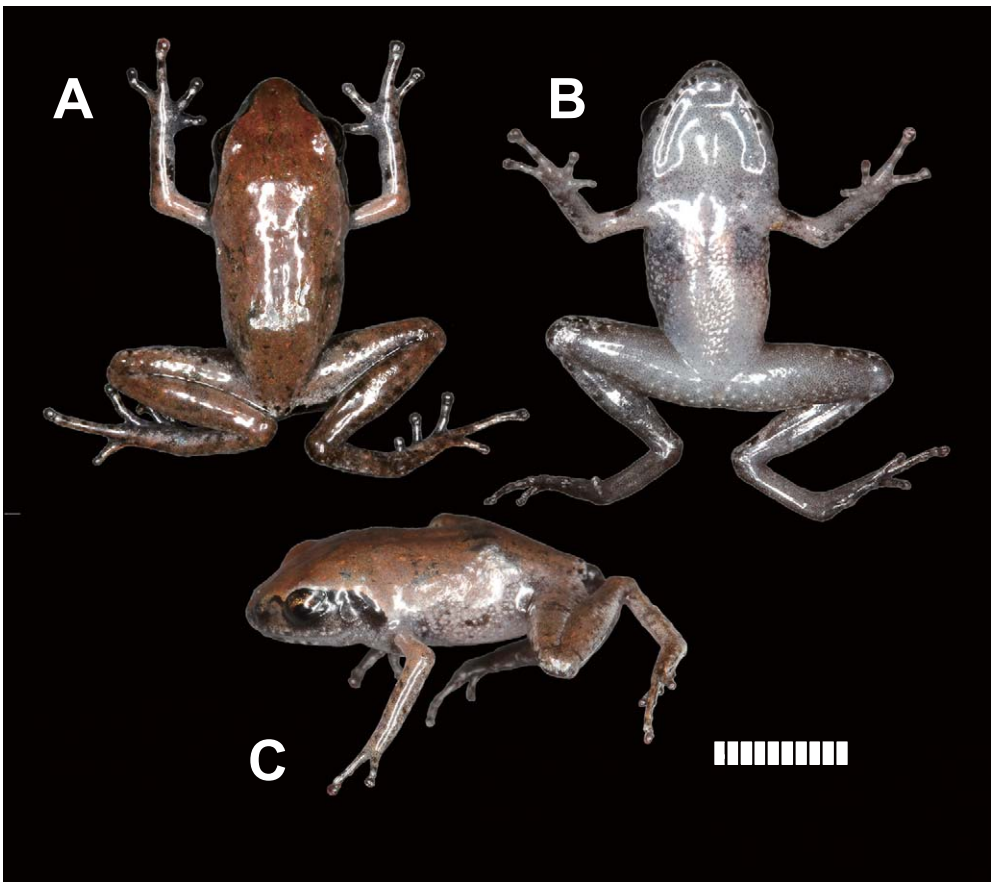

FIG. 4. (A) Dorsal, (B) ventral, and (C) lateral views of juvenile holotype (VNMN 3684) of *Liuixalus catbaensis* sp. nov. in an anesthetized condition. Scale bar=10 mm.

brown stripe on supratympanic fold between eye and arm insertion, including posterodorsal one-third of tympanum and continuing to dark mark at base of upper arm; dorsal red fading to ventral gray at side of trunk, where several irregular dark markings are scattered (Fig. 4C); forelimb and hindlimb dorsally brick-red with faint dark brown bars on forearm and hindlimb; black mark at groin extending to base of thigh; thigh posteriorly dark brown, scattered with white posteroventrally and forming black marking below cloaca; distal half of toe tips darker; ventral side grayish white sparsely dotted with melanophores, more densely on throat than on abdomen (Fig. 4B); iris golden with black reticulations. In preservative, pattern has not obviously changed, although all dorsal surfaces are darkened.

Range

Known from the type locality, Ao Ech (Frog Pond) within the Cat Ba National Park, Cat Ba Island, the largest island of the Cat Ba Archipelago in Ha Long Bay, northern Vietnam (Fig. 1).

FIG. 5. Ventral views of left (A) hand and (B) foot of juvenile holotype (VNMN 3684) of *Liuixalus catbaensis* sp. nov. after preservation. Scale bar= 3 mm.

Natural history

In the type locality, Cat Ba National Park, the holotype was found during the daytime, in a disturbed primary forest surrounded by large limestone rocks. There was a small pool formed in a small stream (ca. 1 m wide) in the forest, near which the frog was found. The air temperature at the time of finding was not recorded, but was 27C at 19:40 h. At night *Hylarana guentheri* (Boulenger, 1882), *Microhyla fissipes* Boulenger, 1884, *Microhyla heymonsi* Vogt, 1911, *Polypedates mutus* (Smith, 1940), and *Chiromantis hansenae* (Cochran, 1927) were found there, but no additional specimens of the new species were encountered.

Comparisons

Because only a single juvenile specimen is available for *Liuixalus catbaensis*, it is impossible to compare its adult body size with those of congeneric species. However, the new species is easily distinguished from *L. romeri* from Hong Kong and Guangxi, China, *L. ocellatus* from Hainan Island, and *L. hainanus* from Hainan Island by: having a dorsally and laterally rounded snout, whose length is slightly longer than the length of the eye (vs. snout slightly constricted in *L. hainanus* and tip bluntly pointed in *L. romeri*, snout shorter than eye in *L. ocellatus* and *L. hainanus*), tympanum diameter about one-third of eye length and separated from eye by one-third of tympanum diameter (vs. tympanum diameter about half eye diameter in *L. romeri* and *L. hainanus*, distance between tympanum and eye about half tympanum diameter in *L. romeri*), hand and forearm 49% of body length (vs. about 40% in *L. romeri*), no webbing between fingers (vs. slight webbing between outer two fingers in *L. romeri*), tibiotarsal articulation reaching between eye and nostril (vs. articulation reaching to anterior corner of eye in *L. ocellatus*, to nostril or snout tip in *L. romeri*, and exceeding snout tip in *L. hainanus*), heels touching each other

when thigh and tibia placed at right angle to body (vs. heels remarkably overlapping in *L. hainanus*), webbing between fourth and fifth toes not reaching to third articulation of fourth (vs. reaching to second articulation in *L. ocellatus*), outer metatarsal tubercle absent (vs. tubercle small or indistinct in *L. romeri*, and small in *L. ocellatus*), dorsal surface nearly smooth (vs. skin with a few tubercles in *L. romeri* and relatively rough, scattered with large and small tubercles in *L. hainanus*) with only sparsely scattered, minute, blunt tubercles between shoulder and sacral regions (vs. upper eyelid with tubercles relatively clear in *L. ocellatus* and numerous in *L. hainanus*, and a pair of small, black, rounded warts posterior to eye in *L. ocellatus*), dorsal ground color brick-red with a few, vaguely defined small brown spots (vs. ground color mostly olive brown or light reddish brown in *L. romeri*, yellowish, reddish, or dark brown in *L. ocellatus*, and brown in *L. hainanus*, with dark horizontal or triangle mark between eyes and a pair of X-shaped dark marks on shoulder, followed by a reverse-V mark in *L. romeri*, a pair of reverse triangular or V-shaped dark markings between eyes, posteriorly with a pair of small black marking in *L. ocellatus*, and with irregular or X-shaped blackish brown markings, medially with a clear, light brown elliptical mark, followed by a triangular dorsal brown mark in *L. hainanus*), and grayish white ventrum sparsely dotted with melanophores (vs. ventrally cream white with gold-yellow tinge, scattered with several dark spots in *L. romeri*).

Discussion

The family Rhacophoridae is the most diverse group of amphibians in Vietnam with a total of 10 genera and 61 species recognized so far (Orlov, 2012; Rowley et al., 2010, 2011b; Nguyen et al., 2013; Nguyen, unpublished data). Indeed, the increase in the number of Vietnamese amphibian species is mainly due to the discovery of new rhacophorids. The description of recent new species is usually made based on molecular and acoustic data (e.g., Rowley, 2011a, b). In the case of the new species described here, only a single juvenile is available and biological data except other than molecular data are insufficient. However, the molecular, as well as morphological, data obtained clearly demonstrate its distinct species status.

The discovery of *Liuixalus* from northern Vietnam is not surprising, since the region is close to southern China, where the genus was restricted previously. In a megophryid frog, *Leptobrachium* (*Vibrissaphora*) *chapaense* (Bourret, 1937), some Vietnamese populations proved to be conspecific with *L.* (*V.*) *hainanense* Ye and Fei in Ye, Fei, and Hu, 1993 from Hainan Island by Matsui et al. (2010), who estimated their separation at 2.6 MYBP, conforming to the date of separation of these regions in the early Pleistocene. Genetic distances on which this dating was based were 1.8–3.0% in 12S and 16S rRNA genes (Matsui et al., 2010). Compared with these values, distances obtained for 16S rRNA genes between *Liuixalus catbaensis* and *L. romeri* from Jinxiu are small (1.8%), suggesting their separation later than in *L.* (*V.*) *hainanense*. However, 12S and 16S rRNA genes on which dating for *Leptobrachium* (*Vibrissaphora*) was based in Matsui et al. (2010) would generally give lower values than 16S rRNA gene alone. Apart from this, it is noted that interspecific genetic distances reported for *Liuixalus* are very small, even smaller than in other rhacophorine genera (e.g., Li et al., 2008, 2013; this study).

The family Rhacophoridae is split into two subfamilies, Buergeriinae Channing, 1989 and Rhacophorinae Hoffman, 1932 (Channing, 1989). Members of Buergeriinae are restricted to the eastern periphery of the Asian continent. Within the Rhacophorinae, *Liuixalus* is now generally considered as the sister taxon of all the remaining rhacophorines (Li et al., 2008, 2009; Yu et al., 2008, 2009; Pyron and Wiens, 2011; Hertwig et al., 2012). Meanwhile the range of the genus is restricted to southeastern China from Hong Kong through Guangxi to Hainan, and now northern Vietnam, all in

the eastern periphery of the Asian continent. Although the origin and subsequent evolutionary history of the Rhacophoridae are in debate (e.g., Bossuyt and Milinkovitch, 2001; Li et al., 2013), the fact that phylogenetically very old groups, subfamily Buergeriinae and the genus *Liuixalus*, occur on the eastern periphery of the Asian continent is biogeographically interesting.

In spite of its remarkable phylogenetic antiquity, the degree of genetic divergence within the genus *Liuixalus* is very small as shown above. This low genetic diversity is suspected to be the result of past population bottlenecks. Probably, after very old separation from the ancestral stock leading to the other rhacophorine genera in the Eocene (around 50.0-36.9 MYBP: Li et al., 2013), the ancestors of *Liuixalus* might have experienced very limited range expansion, severe range fragmentation, and local extinctions before much more recent speciation events occurred at 8.3-3.5 MYBP in the late Miocene (Li et al., 2013). In order to test this hypothesis, further analyses including additional specimens, especially adults, of *L. catbaensis* from Vietnam are necessary.

Acknowledements

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